"INCIDENCE OF ENTEROINVASIVE ESCHERICHIA COLI IN CHILDREN WITH DIARRHOEA AND EVALUATION OF APYRASE BASED -COLORIMETRIC ASSAY FOR ITS EARLY DIAGNOSIS"

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BRANCH – IV

MADRAS MEDICAL COLLEGE
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CERTIFICATE

This is to certify that this dissertation titled **"INCIDENCE OF ENTEROINVASIVE ESCHERICHIA COLI IN CHILDREN WITH DIARRHOEA AND EVALUATION OF APYRASE BASED -COLORIMETRIC ASSAY FOR ITS EARLY DIAGNOSIS"** *is a bonafide record of work done by* ***Dr. M.P. SARASWATHY,*** *during the period of her Post graduate study from June 2006 to March 2009 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai-600003 in partial fulfillment of the requirement for* ***M.D. Microbiology*** *degree Examination of The Tamilnadu Dr. M.G.R. Medical University to be held in March 2009.*

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DECLARATION

I declare that the dissertation entitled **"INCIDENCE OF ENTEROINVASIVE ESCHERICHIA COLI IN CHILDREN WITH DIARRHOEA AND EVALUATION OF APYRASE BASED -COLORIMETRIC ASSAY FOR ITS EARLY DIAGNOSIS"** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***December 2006–May 2008*** *under the guidance of Prof.****Dr.G.JAYALAKSHMI, M.D.,D.T.C.D.,*** *Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Branch IV (Microbiology) examination to be held in March 2009.*

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INTRODUCTION

"The study of disease is really the study of man and his environment"

-Park

Diarrhoea is a universal human experience. Diarrhoea is a symptom, not a disease in itself and it therefore may occur in a wide variety of conditions. In developing countries it is most commonly due to infectious cause[22].

Acute infectious diarrhoea is one of the major causes of mortality in children, particularly in developing countries[33]. Diarrhoeal disorders account for a large proportion (18%) of childhood deaths, with estimated 1.8 million deaths per year globally (World Health Organization 1999)[110]. Also diarrhoeal illnesses may have a significant impact on psychomotor and cognitive development in young children. The WHO suspects that there are more than 700 million episodes of diarrhoea annually in children less than 5 years of age in developing countries. Mortality due to diarrhoea was approximately 3.3 million annually in the 1980s (World Health Organization 2006)[111]. Currently, diarrhoea has been reported to account for 1.6-2.5 million deaths annually [77].

Persistently high rates of diarrhoea among young children despite intensive efforts at control are of particular concern (WHO 2006).[105] Infectious agents are mainly bacterial, viral, protozoal, and rarely fungal. *Escherichia coli (E.coli)* is one of the important causes of enteric infections. These are members of the Enterobacteriaceae family, which are facultative anaerobic gram-negative bacilli, which usually ferment lactose. Most faecal *E.coli* are non-pathogens. However six major groups of diarrhoeagenic E.coli have been characterized on the basis of clinical, biochemical and molecular genetic criteria: 1. Entero toxigenic *E.coli* (ETEC); *2.* Enteroinvasive *E.coli* (EIEC); *3.*Enteropathogenic*E.coli* (EPEC); *4.*Shiga-toxin producing *E.coli* (STEC)/ Enterohaemorrhagic *E.coli* (EHEC)*; 5.*Enteroaggregative *E.coli* (EAEC);

*6.*Diffusely adherent *E.coli* (DAEC).[68]

Enteroinvasive *Escherichia coli* (EIEC) and all four species of Shigella are capable of causing bacillary dysentery. It is a major health problem throughout the developing world that contributes significantly to the loss of 4.6 million lives each year due to diarrhoeal diseases. Bacteriological identification of *E.coli* in stool or environmental specimens requires 48-72 hours; further identification of EIEC entails the additional use of an expensive and time consuming animal assay for virulence[60].

Though molecular methods are sensitive and rapid, they are highly expensive and need considerable expertise which is not available in many laboratories in our country.

Therefore in conjunction with oral rehydration therapy, the development of a simple, inexpensive and reliable method for the early diagnosis of EIEC would reduce the duration and severity of diarrhoea and dysentery by early institution of antibiotic therapy. Indiscriminate use of antibiotics can also be avoided, since it leads to the development of antimicrobial resistance. This study describes the newly developed colorimetric assay that will be useful for specific and early detection of EIEC and *Shigella* species using readily available indigenous component reagents and equipments.

REVIEW OF LITERATURE

Diarrhoea is defined as the passage of loose, liquid or watery stools. Liquid stools are passed more than three times a day and it is a recent change in consistency and character of the stool rather than the number of stools that is more important (WHO 1985)[109]. Dysentery is defined as diarrhoea associated with blood and leukocytes in the stool[103].

Diarrhoea may be further classified as 'acute' if it lasts for less than 2 weeks, 'persistent' if the duration of diarrhoea is 2-4 weeks and 'chronic' if more than 4 weeks duration[84].

Acute diarrhoea may be due to following reasons[41]

1. Infectious	90 %
2. Drug induced	10 %
3. Ingestion of toxins	
4. Ischemia	
5. Other conditions	

Infectious diarrhoea may be caused by viruses, bacteria and protozoa. These agents of diarrhoea are acquired by ingestion of contaminated food or water and transmitted by faeco-oral route[105].

The bacterial agents of diarrhoea can be classified into 2 groups[41].

i) Invasive micro organisms and

ii) Non invasive or Toxin producing organisms

INVASIVE	NONINVASIVE
Enteroinvasive *E.coli* [EIEC]	Enterotoxigenic *E. coli* [ETEC]
Salmonella	Enteropathogenic *E.coli* [EPEC]
Shigella	*Vibrio cholera O1 and non O1*
Campylobacter	*Clostridium perfringens type A*
Enterohaemorrhagic *E.coli* [EHEC]	*Staphylococcus aureus*
Yersinia enterocolitica	*Bacillus cereus*
Yersinia pseudo tuberculosis	*Clostridium difficile*
Vibrio parahaemolyticus	*Shigella dysenteriae type 1*
Edwardsiella tarda	Enteroaggregative *E.coli* [EAEC]
Plesiomonas shigelloides	

Enteroinvasive *E.coli* constitutes one of the important causes of invasive diarrhoea in tropical regions, particularly where sanitation facilities are limited[68].

GENUS DEFINITION:

Escherichia coli (E.coli) is member of Enterobacteriaceae family. *E.coli* is gram negative, non-spore forming bacilli that grow both aerobically and anaerobically on ordinary laboratory media. It is oxidase negative and catalase positive; ferments glucose with production of acid or acid and gas;

reduces nitrates to nitrites and is motile with peritrichous flagella[105].

E.coli is methyl-red positive and ferments mannitol and mannose. It is generally urease negative, does not utilize citrate. It neither produces hydrogen sulphide in triple sugar iron agar nor grows in potassium cyanide (KCN) medium. Enteroinvasive *E.coli* is very similar to *Shigella* species in clinical, pathological features and bio-chemical reactions. It resembles *Shigella* in that most strains are lysine decarboxylase negative, non-motile, late-lactose or non-lactose fermentors and anaerogenic[59].

HISTORICAL REVIEW

Escherichia coli was first identified by Theodor Escherich, German pediatrician while studying on the intestinal flora of infants. He described the organism in 1885 as bacterium coli commune and established its pathogenic properties in extra intestinal infection. The first identification of *E.coli* that could cause diarrhoea was made in the United Kingdom on the basis of epidemiological investigations of outbreaks of community acquired and nosocomial infantile gastroenteritis in 1945. *E.coli* has been studied to such an extent that it is now the most thoroughly understood free-living live form on earth[105].

EIEC strains were first shown to be capable of causing diarrhoea in volunteer studies conducted by DuPont et al. in 1971. EIEC are very similar to *Shigella* species in every respect except for their lower acid resistance which

results in higher infectious inoculums approximately 10^8 bacteria/ ml and their inability to produce shiga toxin which may be related to the fact that so far no complications such as Hemolytic Uremic Syndrome (HUS) have been observed in EIEC infections[23]. These 'atypical' E.coli strains had earlier been grouped under the 'Alkalescens-Dispar Group'.

Compelling evidence indicates that Shigella species and EIEC are derived from multiple origins of *E. coli* and form a single pathovar. EIEC strains are regarded as precursors of 'full-blown' Shigella evolved from E. coli. By gain and loss of functions, *Shigella* and EIEC became successful human pathogens through convergent evolution from diverse genomic background[79].

HABITAT

E.coli is part of the normal intestinal flora of both humans and warm-blooded animals. The organism is excreted in faeces and may survive in the environment; however, it appears that there is no independent existence outside the body. Thus *E.coli* is considered an indicator organism for faecal contamination and is an important parameter in food and water hygiene. The organisms are transmitted by faeco-oral route or via contaminated food and water[105].

CLASSIFICATION OF ENTEROVIRULENT E.COLI

Based on the Clinical, biochemical, molecular characteristics and their pattern of interaction with epithelial cells and tissue culture monolayer,

diarrhoeagenic *E.coli* is classified as[71]

1. Enterotoxigenic *E.coli* (ETEC)
2. Enteropathogenic *E.coli* (EPEC)
3. Enterohaemorrhagic *E.coli* (EHEC)
4. Enteroinvasive *E.coli* (EIEC)
5. Enteroaggregative *E.coli* (EAEC) and
6. Diffusely Adherent *E.coli* (DAEC) [68]

In addition to the 6 classes of diarrhoeagenic *E.coli* mentioned above, there are other potential classes, such as ‘’cytolethal distending toxin (CDT) producing *E.coli* and cell detaching *E.coli* (CDEC) that have yet to be fully characterized[68].

MORPHOLOGY

E.coli is a rod shaped cell of 2-6 µm in length and 1.1-1.5 µm in width with rounded ends[75]. The shape may vary from coccal to long filamentous rods[43]. By Gram’s stain, it appears as gram-negative rods with uniform staining. It does not form spores. It is usually motile by peritrichous flagella, except the “inactive” biovar of *E.coli*[57, 99]. It possesses a capsule made of acidic polysaccharide[74]. It produces different kinds of fimbriae that vary in structure and antigenic specificities[74].

METABOLISM AND GROWTH REQUIREMENT

E.coli is an aerobic, facultative anaerobic organism. It is a chemo-

organotroph, having both respiratory and fermentative type of metabolism, but the growth is less copious under anaerobic conditions. E.*coli* exerts pronounced metabolic activity between 15˚C and 45˚C[43]; under optimum conditions the generation time is 20 minutes.

The optimum temperature is 37˚C at which they grow well on ordinary media containing 1% peptone[44]. In contrast to most other coliform bacteria, *E.coli* ferments lactose and produces indole at 44˚C that is used for identification in food and water bacteriology. *E.coli* is more heat resistant than most other species of Enterobacteriaceae and survives at 60 ˚C for 15 minutes or 55 ˚C for 60 minutes. It is sensitive to brilliant green and 0.25% deoxycholate[43].

Escherichia strains can be maintained for years in tightly closed nutrient agar stabs or on Dorset egg medium kept in the dark at room temperature. However, even without further subcultures such strains tend to mutate to the R forms and to lose virulence plasmids. Cultures can be preserved in tryptic soy broth containing 20-40 % glycerol at -70˚C or in liquid nitrogen[74].

STRUCTURAL FEATURES

Inner membrane

The inner or cytoplasmic membrane, which is impermeable to polar molecules, regulates the passage of nutrients, metabolites, macromolecules

and information in and out of the cytoplasm and maintains the proton motive force required for energy storage[61].

Periplasmic space

A variety of functional categories of proteins are found in the periplasm[73] including an Apyrase enzyme – a novel variant of acid phosphatase.[50]

Peptidoglycan cell wall

It is composed of a thin layer of Peptidoglycan, with a short peptide and D-alanine attached to the carboxyl group of muramic acid. The envelope is responsible for the shape and osmotic stability of the organism[78].

Outer membrane

The outer membrane is an asymmetrical lipid bilayer. Phospholipids occur almost exclusively in the inner layer; whereas the outer layer is composed of peptidoglycan. It plays a major role in protecting the bacteria from various detergents, dyes and hydrophobic antibiotics[72].

Lipopolysaccharide (LPS)

The LPS is an extremely potent virulence factor of EIEC. The common serogroups of EIEC have LPS antigens related to the Shigella LPS. It has three major domains, the lipid backbone, the core phosphorylated

oligosaccharide, and the repeating oligosaccharide side chains. Lipid A, also known as endotoxin, is the biologically active portion of the molecule that is recognized by the host. The repeating oligosaccharide attached to the LPS core is known as O antigen and is the basis for serogroup classification[82].

SURFACE ANTIGENS

The surface antigens consists of the following were useful in serotyping of *E.coli*.

Somatic antigen (O antigen)

There are 173 O groups in *E.coli*. As *E.coli* O antigens are not type or species specific, numerous cross reactions are known between *E.coli & Shigella*, *Citrobacter, Salmonella, Providencia* and *Yersinia* [43]. Therefore O-antigen typing must always be associated with a proper biochemical identification of isolates[43]. This is particularly important for *Shigellae* that share partial or identical O antigens with *E.coli.* The following table shows the example of identical O antigens in EIEC and *Shigellae*[71].

Antigenic sharing with Shigella

EIEC –O antigen	Shigella -corresponding O serovar
O 112 ac	O2 S.dysenteriae
O124	O3 S. dysenteriae
O152	O12 S.dysenteriae
O143	O8 S.boydii

Capsule (K antigen)

These antigens are belonging to class I capsular polysaccharides[48]. Capsules widely vary in chemical structure and are the basis of the K-antigen

serotyping scheme. There is over 100 K-antigen types in *E.coli.*

Fimbria (F antigen)

Certain fimbrial antigens are involved in the adhesion process and therefore are important virulence factors. They develop at 37˚C but not at 18 ˚C. They are heat labile proteins. Fimbrial antigens agglutinate a variety of erythrocytes that can be used for their characterization[75].

Flagella (H antigens)

There are 56 H-antigens available in *E.coli*. They serve several functions, including roles in adhesion and genetic exchange through conjugation[29].

EPIDEMIOLOGY OF ENTEROINVASIVE E.COLI

Epidemiologic studies of EIEC mostly describe outbreaks. In sporadic cases, many EIEC strains are probably misidentified as *Shigella* species or nonpathogenic *E. coli* strains. It affects all the age groups, but children are more susceptible. Since EIEC has got higher environmental resistance,[52] EIEC outbreaks are usually food borne or waterborne[56, 63,100].although person-to-person transmission does occur[40]. The infective dose of EIEC in volunteers is higher than that for *Shigella* species[24], thus the potential for person-to-person transmission is lessened. Endemic disease occurs in developing countries, where these bacteria can be isolated with relatively high frequency.

Endemicity occurs generally where *Shigella* species are also prevalent, but the epidemiologic features may be different from those of *Shigella* species[52]. In the developing world, as many as 5% of sporadic diarrhoea and 20% of bloody diarrhoea cases may be caused by EIEC strains[68].The incidence of EIEC in developed countries is thought to be low, but occasional food borne outbreaks, do occur[80].

VIRULENCE FACTORS

Though *E.coli* is a commensal intestinal flora, it is considered as one of the primary enteric pathogens. Pathogenic strains posses special virulence factors that are usually absent in commensal *E.coli*. There is general conceptual agreement on the principle of "Molecular Koch's postulates" as articulated by Stanley Falkow. By this definition "a trait is considered to be virulence factor if it is found specifically in strains of a microbe that cause disease". Adhesins, enterotoxins, hemolysin, iron acquisition, lipopolysaccharide, capsule and plasmids are some of the virulence factors of *E.coli* [61].

a) Secretion systems and toxins

EIEC can directly interact with host cell through a dedicated type III secretion system (T3SS). They not only export proteins through the inner and outer bacterial membranes, but also inject them into or through the host cell membrane[35].

b) Iron acquisition

Iron is an essential element, required by virtually all-living organisms as a cofactor for several indispensable enzymes. Enterobacterial pathogens contain several efficient systems that scavenge iron. Siderophores are low molecular-weight iron chelating molecules that are synthesized, secreted and recaptured by microorganisms. Siderophores are mainly of 2 types

1. The phenolate type e.g. enterobactin
2. Hydroxamate type e.g. aerobactin

Aerobactin is considered to be an important virulence factor for EIEC[101].

c) Plasmids

Enteroinvasion of EIEC is mediated by a 220 mega Dalton high-molecular-weight plasmid [81]. The genes encoded on plasmids may play a major role in pathogenesis. The entire Type III secretion system that endows Enteroinvasive *E.coli* and *Shigella* strains with the ability to invade epithelial cells is encoded by *mxi* and *spa* genes present on a large plasmid[2, 3,108].

d) Apyrase

Apyrase (ATP-diphosphohydrolase) enzyme is found in eukaryotes and some prokaryotes. In prokaryotes, Apyrase has been found only in EIEC and *Shigellae* [94].

Apyrase is a Periplasmic enzyme, present only in virulent strains of EIEC and *Shigella* [53]. It is encoded by *phoN2 (apy)* gene present in a large virulence plasmid and also in the chromosome. Expression of *phoN2 (apy*) is

regulated by the *virF-virB* gene cascade[91]. It has been considered as a potential virulence factor for the following reasons[94, 92].

- Apyrase is involved in the process of mitochondrial damage resulting in death of the EIEC infected host cell.
- Recently it has been shown to be involved in intracellular spread of the pathogen. The ability of EIEC to move within the cytoplasm of the infected cells and to spread to adjacent cells solely relies on the production and localization of Ics A protein at one pole of the bacterial surface. The apyrase is responsible for this unipolar localization of Ics A protein[92].
- It also plays a major role in inducing condensation of the host actin to form an actin tail by Ics A protein, there by allows EIEC to propel itself through the cytoplasm of infected cells to adjacent cells[92].

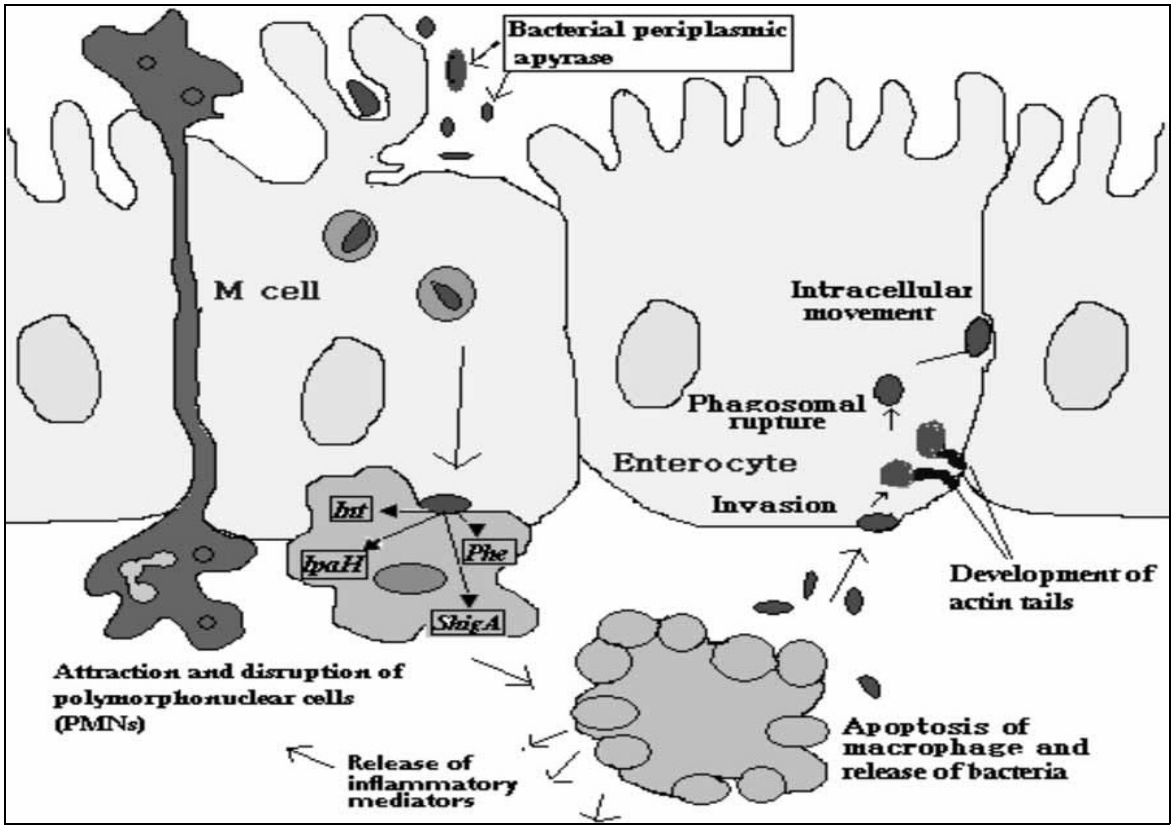
The following Virulence genes are responsible for pathogenesis of EIEC infection [105].

Virulence Genes	Location	Putative role of gene product in disease
ipa D	p	Attachment to host cell, induces rearrangement of cytoskeleton and phagocytosis
ipa B ,ipa C	p	Induction of phagocytosis , lysis of phagosome after which cells reach the cytoplasm
Mxi	p	At least 10 different loci; involved in export of *Ipa* proteins
Ics A, ics B	p	Intercellular spread between adjacent cells
vir F	p	Transcriptional activator of *vir B*

vir B	p	Transcriptional activator of *ipa* gene family *(ipa A, ipa B, ipa C, ipa D)*, regulate the expression of phoN2 gene which codes for the Apyrase enzyme[92].
Vir R	c	Negative regulator of *vir F* ; probably mediates temperature control of virulence expression
vac C ,vac B, ksp A	c	Regulate *ipa* gene family and *ics A*
Omp R , env Z	c	Osmoregulation of virulence genes located on plasmids
Ipa H, ial	p	Used as a target genes for PCR

c- Chromosome p- Plasmid

EIEC PATHOGENESIS



Courtesy: A.R. Pavankumar and K. Sankaran: Tools for surveillance of *Escherichia coli*, *Food Technol. Biotechnol. 46* (2)125–145 (2008) 129.

PATHOGENESIS AND PATHOLOGY

EIEC enters the phagocytic 'M' cells (micro fold cells), which are

specialized epithelial cells overlying mucosal lymphoid follicles, and are taken up by macrophages [90]. However, they escape the phagosome and trigger apoptosis of the macrophage and are released. They enter adjacent cells by binding to host protein called integrins[9, 46], located at the basolateral surface and thereby invoking phagocytic uptake by these cells[88]. Meanwhile, these events trigger the host inflammatory response, attracting polymorphonuclear cells that migrate to the luminal side, destroying tight junctions between the epithelial cells. Once EIEC enters the host cell; a complex array of proteins induces condensation of host actin to form actin tail by the protein IcsA [1, 107], which allows EIEC to propel itself through the cytoplasm to adjacent cell[9, 36]. They replicate therein, resulting in necrosis and stripping of large areas of colonic mucosa and dysentery similar to but usually less severe than Shigella dysentery [18, 42]. The inflammation decreases colonic reabsorption resulting in diarrhoea[4]. EIEC causes colonic lesions with ulcerations, hemorrhage, mucosal and sub mucosal edema with infiltration by polymorphonuclear leukocytes[71].

The expression of invasiveness is complex. The invasion related plasmid has been designated as *pInV* [6], where the genes required for bacterial entry into epithelial cells are clustered. Prominent among these genes are the *mxi* and *spa* loci, which encode a so-called type III secretion apparatus. This machinery is required for the secretion of multiple proteins which are necessary for full pathogenicity. Among these proteins, the invasion plasmid antigens *Ipa* A, B, C & D are necessary for invasion [65]. Other proteins

including the enzyme apyrase play a role in the ability of EIEC to move within the cytoplasm of infected cells and to spread to other cells[66].The *ipa H* and *ial* genes were used as a target for PCR to detect EIEC from stool samples.[71]

CLINICAL FEATURES

Infection usually is confined to the gastrointestinal mucosa[83]. The ability of EIEC to invade and colonize the intestinal epithelium is a key determinant of the disease. Infection presents most commonly as acute watery diarrhoea, indistinguishable from ETEC[68]. It progresses to dysentery characterized by severe cramps, fever, tenesmus, frequent passage of small volume of stool with mucus and blood[103]. It affects all the age groups. Their inability to produce shigatoxin may be related to the fact that so far no complications such as Hemolytic Uremic Syndrom have been observed in EIEC infections[106].

TYPING METHODS

There are numerous methods available for sub typing *E.coli*. Each method has advantages and disadvantages when applied to a specific situation.

1. Conventional typing methods
    - Biotyping[19]
    - Resistotyping[105]
    - Serotyping[75]

2. Molecular methods

- Plasmid profile[12]
- Restriction endonuclease digestion and gel electrophoresis[105]
- DNA hybridization-Southern blot analysis and colony blots of bacterial cultures[39]
- Randomly amplified polymorphic DNA[14]
- Pulsed field gel electrophoresis[67]

Biotyping is the most widely used typing method to characterize E.coli. Ornithine decarboxylase, Lysine, Mucus, Motility and gas production can be used for initial detection of EIEC strains[32, 103]. Resistotyping may provide a first hint for a common origin of 2 or more strains.

Conventional typing methods have been succeeded in recent years by molecular techniques that are applicable to a wide variety of organisms with only minimal adaptation in the procedures. These newer methods are often more discriminatory but technically more demanding and require specialized equipments.

SEROLOGICAL ANALYSIS

Serotyping of *E. coli* occupies a central role in the history of these pathogens. Prior to the identification of specific virulence factors, serotyping was the predominant method by which this organism was identified in the

laboratory. In 1944, Kauffman proposed a scheme which classified *E.coli*, the modified form of which is still in use today.

E.coli comprises of many serotypes. A combinations of O (cell wall polysaccharide), H (flagellar proteins), K (capsular polysaccharide or envelope) antigens, which are identified with antisera raised against 173 O antigens, 56 H antigens and more than 100 K antigens. A specific combination of O and H antigens defines the serotype of an isolate. The most common serotypes associated with Enteroinvasive *E.coli* are O28ac, O29, O112ac, O124, O136, O143, O144, O152 and O164.[51]

LABORATORY DIAGNOSIS

COLLECTION AND TRANSPORT OF THE FECAL SPECIMENS:

The stool specimens should be collected in a clean, wide mouthed container covered with a tight fitting lid. Rectal swabs may be collected in newborn and severely debilitated adults. The samples should be transported immediately to the laboratory; if a delay longer than 2 hours is anticipated the specimen should be placed in a transport medium. Glycerol phosphate buffer supports viability of EIEC better than other media[54].

CULTURE CHARACTERISTICS

Enteroinvasive *E.coli* strains grow well in ordinary media containing 1% peptone. The use of Xylose Lysine Deoxycholate in combination with macConkey or Eosine Methylene Blue agar provides the best chance of

recovery of EIEC from faecal samples[102]. 5-10 suspicious colonies may be selected for definitive biochemical reactions, combined with agglutination with group or serovar specific antisera.

Nutrient agar:

After 18-24 hrs of incubation, large (2-3mm), circular, glistening, smooth, convex and non-pigmented colonies are produced by smooth forms whereas rough form produces dry, wrinkled, rough colonies[105].

MacConkey agar

Colonies are pink lactose fermenting or non-lactose fermenting colonies as in the case of atypical biovar[105, 54].

Xylose-Lysine Deoxycholate agar

Yellow or translucent colonies without black center (Hydrogen sulphide) are produced[105].

Liquid media

In liquid media such as peptone water, smooth forms show homogenous turbid growth after 12-18 hours, whereas rough forms agglutinate spontaneously, forming a sediment on the bottom of the test tubes. Pellicle formation on the surface of liquid media can be seen in heavily fimbriated strains after prolonged incubation for more than 72 hrs at 37˚C[105].

Biochemical Characteristics

The following table shows the differentiating biochemical features of E.coli and Shigella

+, ≥90% positive; -, ≤10% positive; v- variable, 11-89% positive; +a - *Shigella dysentriae* always negative[31].

Tests for confirmation

EIEC strains are confirmed by the following tests for invasiveness.

a. Sereny's test

The test assesses the ability of EIEC to cause an ulcerative keratoconjunctivitis after instillation onto the cornea of a guinea pig/rabbit[95]. The animal is examined daily for the development of keratoconjuncivitis for 7 days. There is cell destruction and superficial inflammatory invasion of the cornea similar to that in colonic mucosa. The disadvantage of the test is the cost of animal; painful technique and objections often made on humane grounds[61].

b. Tissue culture test

Tissue culture study is done to detect the ability of EIEC strains to invade intestinal epithelial cells. The invasive phenotype of the bacterial isolates is confirmed by invasiveness in a confluent monolayer of HeLa cells/ Hep2

cells[67]. Hep2 monolayer is overlaid with 2ml of the bacterial suspension containing 10^7 organisms/ml. After incubation at 37°C for two and a half hours, the monolayer is washed, fixed and stained with Giemsa. The monolayer is examined under oil immersion by light microscope for the presence of intracellular organisms and plaque formation. Tissue culture test is a better alternative to the sereny test as it gives more detailed study of the invasive mechanism[20].

c. Congo red binding assay

Congo red binding (CRB) capacities are encoded by 220 kb virulence plasmid; hence virulence property of EIEC is well correlated with CRB[76]. The virulent strains can be identified by the appearance of red colonies on Congo red dye agar[60].

d. Virulence marker antigen (VMA) ELISA

The plasmid codes for outer membrane antigens called Virulence marker antigen can be detected by the ELISA test[16, 60].

e. Colorimetric assay

The simple test detects the enzyme Apyrase, which is specific for EIEC and *Shigella*. The test uses sodium pyrophosphate as a substrate for the enzyme. EDTA used in the test acts as a buffer and a metal chelator to suppress other phophatase and pyrophophatase activities that are present in the stool. The released monophosphate can be detected by adding ammonium molybdate and ferrous ammonium sulfate, with which it react to form

molybdenum blue. This results in a change of color to blue. This can also be read with a spectrophotometer to find out the optical density value[55]. The test can not differentiate EIEC and *Shigella*. However, for clinical laboratories, determining the presence of virulence factors will be more meaningful than genus level identification[105].

This test detects the Apyrase enzyme in 8 hours. Less technical expertise is required for this test, when compared to the methods currently available for the diagnosis of EIEC. Moreover, multiple samples can be tested simultaneously and results can be interpreted by using color card or colorimeter. Hence it can be used during epidemics of EIEC infection where large numbers of cases have to be screened, where the expensive equipments cannot be accessed. It assists in the early institution of antimicrobial therapy, as the results can be obtained on the same day.[55]

Apyrase is being chosen as a diagnostic target because[5,55]

- It is specific to EIEC and *Shigella*
- It can be assayed by a simple and sensitive colorimetric method
- Its surface accessibility minimizes sample handling and processing
- Its ability to act both on organic and inorganic pyrophosphates in the absence of metal ions makes it possible to detect it in the presence of other cellular phosphates and pyrophosphates, which invariably require metal ions[5].

f. Polymerase chain reaction for EIEC

PCR involves the enzymatic amplification of DNA in vitro. This method

is capable of increasing the amount of the target DNA sequence in a sample by synthesizing many copies of DNA segment. PCR is carried out in discrete cycles and each cycle of amplification can double the amount of target DNA. The target DNA is exponentially amplified so that after 'n' cycles there are 2n times as much target DNA as was present initially.

PCR targeting virulence associated gene sequences can be performed on colony sweeps from MacConkey agar or other moderately selective agar plates. Ideally such a procedure should be followed by subculture and identification of the responsible pathogenic strain[105].

Direct PCR with stool samples has also been developed, which targets invasion plasmid antigen H (*ipaH*) gene sequences. This PCR system is very effective for diagnosing cases of dysentery. Direct PCR on stool samples help in rapid diagnosis; but it could not differentiate EIEC from *Shigella*[97].

PCR using *ial* probe is virtually 100% sensitive and specific for EIEC strains that have retained their virulence[87]. The *ial* PCR is also effective in a multiplex PCR system to identify EIEC strains simultaneously with other *E. coli* categories. *phoN2 (apy)* genes present on 220MDa large virulence plasmid can also be used as a target for PCR[50].

ANTIMICROBIAL RESISTANCE

The expression of one or more antibiotic resistances by the majority of the EIEC strains raises the potential for problems when treating diarrhoea

and dysentery produced by EIEC[39].

EXTENDED SPECTRUM β-LACTAMASE PRODUCTION

The treatment of *E.coli* infection is increasingly becoming difficult because of the multi drug resistance exhibited by the organisms. ESBLs are increasingly found in *E.coli*. These are plasmid mediated β-lactamases which have the ability to hydrolyze β-lactam antibiotic containing oxyimino group. ESBLs have serine at their active site and attack the amide bond in the lactum ring of antibiotics causing their hydrolysis[15].

ESBLs can be detected by the following tests[15]

1. Double disc synergy test
2. Three dimensional test
3. Inhibitor potential disc diffusion test
4. Disc approximation test
5. MIC reduction test
6. E test
7. Molecular methods

TREATMENT

The broad principles of management of acute gastroenteritis in children include oral rehydration therapy, enteral feeding and diet selection, Zinc supplementation and additional therapies such as probiotics. Early diagnosis

and timely antibiotic therapy in EIEC infection may shorten the duration of illness and prevents complications. Trimethoprime-sulfamethoxazole is an appropriate choice of antibiotic if the organisms are susceptible[71].

PREVENTION

Preventive strategies includes

1. Promotion of exclusive breast feeding
2. Improved complimentary feeding practices like Vitamin A supplementation reduces childhood diarrhoea by 34%.
3. Improved water and sanitary facilities and promotion of personal and domestic hygeine.
4. Behavioural change strategies through hand washing promotion and access to soap reduce the burden of diarrhoea in developing countries[71].

AIMS OF THE STUDY

- To study the incidence of Enteroinvasive *Escherichia coli* infection in children with diarrhoea and dysentery
- To evaluate colorimetric assay for Apyrase enzyme as a diagnostic tool in the early diagnosis of EIEC diarrhoea
- To compare colorimetric assay with Polymerase Chain Reaction and Sereny's test
- To determine the serotype of the EIEC isolates
- To determine the antimicrobial susceptibility pattern of the isolates
- To estimate the prevalence of Extended spectrum β-lactamase production among the EIEC isolates

MATERIALS AND METHODS

PERIOD OF STUDY:

December 2006 to May 2008.

PLACE OF STUDY:

This cross sectional study was conducted at the Institute of Microbiology, Sample were collected from Institute of Child health Madras Medical College and Government General Hospital, Chennai-3.

SELECTION OF STUDY GROUP[87]

A total of 145 children, 0-5 years of age were included in the study. The patients were clinically examined and selected for the study after obtaining the history.

Inclusion criteria

Children were included in the study if they had more than three loose stools in 24 hours or an episode of dysentery.

Exclusion criteria

The following children were excluded from the study

1. Children with diarrhoea due to food intolerance
2. The children who acquired diarrhoeal illness after hospital admission.

SELECTION OF CONTROL GROUP:

A total of 50 children with no history of diarrhea for a period of 2 weeks in the 0-5 years of age were included for comparison with the study group.

ETHICAL CONSIDERATION

Ethical and research clearance was obtained from the Institutional Ethical Committee, Government General Hospital & Madras Medical College, Chennai-3. Permission to conduct the study was sought from the respective hospital authorities. Informed consent was obtained from parents/ guardians of the child before enrolment into the study.

STATISTICAL ANALYSIS

The proportional data of cross sectional study was tested using Pearson's Chi-square(x^2) analysis, Two sample binomial proportion test. Statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) and Epi-info software.

COLLECTION AND TRANSPORT OF STOOL SAMPLES

The stool samples were collected in a clean wide mouthed container transported to the laboratory and processed within one hour[54]. The usage of any transport media was avoided as the phosphate buffer present in the media might alter the results of colorimetric assay.

MICROBIOLOGICAL ANALYSIS

Macroscopic examination:

The stool samples were examined for color, consistency, presence of blood, mucus, segments of parasite and foreign body.

Direct microscopic examination:

Saline, Iodine and Methylene blue stained wet preparations were examined for the presence of erythrocytes, leukocytes, ova and cysts of the parasites.

Isolation and Identification of EIEC

The collected stool samples, if present, mucus or blood stained portions were inoculated onto MacConkey agar agar, Xylose Lysine Deoxycholate agar[102] and SeleniteF broth. The plates were incubated at 37˚C for 24 hours. The selenite F broth was sub-cultured onto XLD agar after 6-8 hours of incubation and these plates were read after overnight incubation. On XLD agar: EIEC produced yellow or pink colonies without black center[54]. On MacConkey agar agar: EIEC colonies appeared as either lactose fermenting or non lactose fermenting; hence from each sample the lactose positive and all lactose negative colonies were subjected to identification procedures[103]. Similar to study group, the stool samples from control children were also processed by standard microbiological procedures.

The following tests were used for identification of E.coli

1. Catalase
2. Oxidase
3. Indole
4. Triple Sugar Iron agar
5. Citrate
6. Urease
7. Mannitol motility medium

The stool samples collected from study and control group were subjected to colorimetric assay to detect the presence of the enzyme Apyrase. The Apyrase positive isolates and E.coli isolates were retested with colorimetric assay after 7 hours of culture in Luria Bertani broth and were subjected to PCR also. Then PCR positive strains were tested for their invasiveness by Sereny's test. The isolates were preserved in Nutrient broth containing 40% glycerol frozen at -70˚C[39].

All the isolates were subsequently tested for antimicrobial susceptibility by Kirby-Bauer disc diffusion method with a battery of 7 different antibiotics. ESBL producers were detected by combined test method.

Sereny's test:

A volume of 25µL containing 10^9organisms/ml was put into the right conjunctival sac of test animal. A 25µL of culture containing 10^9organisms/ml

of *Shigella dysenteriae* was given to the control animal, which served as the positive control. The uninoculated left eye of each animal used in the test served the negative control. Animals were examined daily for 7 days for the evidence of conjunctivitis or keratoconjunctivitis[20].

Serotyping:

The isolates were sent to Centre for Diarrhoeal Diseases Research & Training, Kolkata for serotyping.

ANTIMICROBIAL RESISTANCE

Antimicrobial susceptibility pattern [7]

Mueller-Hinton agar plate was inoculated with 0.5 McFarland standard inoculums to obtain a lawn culture. Using sterile forceps, discs were placed over the agar surface, incubated at 37˚C for overnight. The results were interpreted as per Clinical Laboratory Standards Institute (CLSI) standards. The *E.coli* strain ATCC 25922 was included as a quality control in all tests.

The following antibiotic discs were used[17]:

Drug	Disc content (µg)	Diameter of zone of inhibition (mm)			ATCC E.coli 25922
		R	I	S	
Amikacin	30	14	15-16	17	19-26
Ampicillin	10	13	14-16	17	16-22
Cefoperazone-Sulbactum	75	15	16-20	21	28-34
Cefotaxime	30	14	15-22	23	29-35
Ciprofloxacin	5	15	16-20	21	30-40
Co-trimoxazole	T1.25/S23.75	10	15-Nov	16	23-29
Gentamicin	10	12	13-14	15	19-26

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R: Resistant ; I: Intermediate; S: Sensitive

Extended Spectrum β-lactamase detection[17]

A two step screening and confirmation of ESBL was done.

Step 1- Screening

Cefotaxime and Ceftazidime were used as an indicator drugs. The isolates showing resistance to Cefotaxime (≤22mm) and Ceftazidime (≤18) were considered as suspected ESBL producers[69].

Step 2- Confirmatory test:

Combined disc method:

Mueller-Hinton agar plate was inoculated with 0.5 McFarland standard inoculums to obtain a lawn culture. The discs of ceftazidime (30µg) and ceftazidime-clavulanic acid (30/10 µg) were kept over agar surface. An increase of 5 mm or more in diameter of the zone of inhibition with ceftazidime-clavulanic acid when compared to ceftazidime alone was confirmed an ESBL producer[69, 15].

COLORIMETRIC ASSAY

All the steps were performed using transparent radioimmunoassay (RIA) vials. The positive test results in a change of color to blue. This can also be read with a spectrophotometer to find out the optical density (OD) value and can also estimate the color semi quantitatively against a color card. The OD value of 0.3 was taken as cut-off point. The Apyrase gene, *apy* of *Shigella* was cloned in vector plasmid pRSETB and maintained in *E.coli* strains GJ1158 was used as a positive control and Enteropathogenic *E.coli* was used as a negative control[5, 55].

Materials

1. Centrifuge

2. Vortex

3. Spectrophotometer

4. Reagents

   i. Wash Reagent: 0.9% Saline- 1mM Calcium chloride

   ii. Assay Reagent

      a) Assay buffer: 40mM EDTA, pH 7.5

      b) Substrate Solution: 6mM Sodium Pyrophosphate

   iii. Color Reagent

      a) Reagent A: 5% Acidic Ammonium Molybdate

      b) Reagent B: 1% Ferrous Ammonium Sulphate

a. Treatment of stool samples for detection of EIEC

This following procedure is done for the benefit of simplified inoculation and to minimize pyrophosphatases contamination from stool particles. These insoluble stool materials are retained by the filter paper whereas the organisms pass through and inoculate the media in the vials.

Take Radioimmunoassay (RIA) vials with cap, with 1 ml of Luria Bertani medium.

↓

Pierce the bottom of the caps at four different places with the help of a 25G needle

↓

Place the Whatmann NO.1 filter paper snugly against perforated bottom of the caps

↓

Add 500µl of stool samples into the cap

Seal the caps with a piece of parafilm “M”

↓

Spin the RIA vials at 4000 rpm, for 1 minute, to inoculate the media contained in the vial

↓

The vials are incubated at 37˚C for 7 hours

↓

Then do the assay for the presence of Apyrase.

b. Diagnostic assay protocol for apyrase

Culture grown for 7 hours at 37˚C

↓

Pellet down at 6000 rpm for 3 minutes

↓

Discard the supernatant, add 1 ml of wash reagent and suspend the pellet by brief and gentle vortexing } 2 times

↓

Pellet down at 6000 rpm for 3 minutes

↓

Add 300 µl of assay Reagent and suspend by brief vortexing

↓

Incubate at 37˚C for 15 minutes

↓

Spin at 6000 rpm for 3 minutes and remove 100 µl of supernatant from each sample and transfer to a microtitre plate

↓

Add 200 µl of color Reagent and leave at room temperature for 10 minutes
Read at 650 nm

POLYMERASE CHAIN REACTION [50, 55]

PREPARATION OF CELL LYSATE

Cell lysates of isolates were used as DNA templates for colony lysate PCR. Around 5-10 colonies were suspended in 100 µl of Tris-EDTA buffer and boiled for 5 minutes and rapidly chilled on ice for 5 minutes. After 4 min of centrifugation in a microfuge at 12,000 rpm, 1µl of the supernatant was obtained and used as template.

COMPONENTS OF PCR:

1. Oligonucleotides

The oligonucleotides used in this procedure were only 24 nucleotides in length because they were too short to form stable hybrids at the temperature used for polymerization. Oligonucleotides were used at a concentration of 5 pico moles were sufficient for 30 cycles of amplification.

Oligonucleotides used in this protocol are:

Forward primer--apy-1, 5'GGG AAT TCC ATA TGA AAA CCA AAA A 3'

Reverse primer--apy-4, 5'CCC AAG CTT TTA TGG GGT CAG TTC ATT 3'

2. PCR Buffer

DNAs used as templates were dissolved in low ionic Tris- EDTA buffer. The concentration of Mg^{2+} optimized when ever a new combination of target and primer was used and also when deoxynucleotide triphosphate (d NTP) primers were altered.

3. Taq DNA polymerase

A genetically engineered form of enzyme synthesized in E.coli (Amplitaq) was used. This polymerase enzyme carries a 3' to 5' exonuclease activity. Approximately 2 units of this enzyme were required to catalyze a typical PCR.

4. Deoxynucleotide triphosphate (dNTPs)

Deoxynucleotide Triphosphate (dNTP's) is used at saturating concentration (200µl of each dNTP's). A stock solution of dNTP's (50mM) is

adjusted to pH7 with 1N NaOH to ensure that the pH of the final reaction does not fall below pH7.

PCR REACTION MIXTURE

PCR PROTOCOL

Amplification was carried out in *eppendorf* gradient thermal cycler for 35 cycles with initial denaturation of DNA for 5 minutes at 95˚C, then subsequently for 1 minute at 95˚C for each cycle. Annealing of the primer was done for 1 minute at 55˚C and elongation of chain for 1 minute at 72˚C. After the completion of 35 cycles, a warming up period of 7 minutes was given.

The same composition of PCR mixture was used for all PCR reactions. Native apyrase clone and Enteropathogenic *E.coli* were used as a positive and negative control respectively. The PCR amplification products were run in horizontal 1.2% agarose gel electrophoresis, which was set at 125 V and run for 1 hour. The gel was visualized using a UV transilluminator. PCR products and 100 base pair molecular markers were seen as bright fluorescent adducts due to binding adduction of ethidium bromide to DNA.

INTERPRETATION

In positive amplification, a single PCR product band at 790 base pair was obtained.

RESULTS

- 195 stool samples were collected from children less than 5 years of age which include 145 children with diarrhoea and 50 controls without diarrhoea. The samples were cultured for *Escherichia coli*, and identified by standard microbiological procedures such as colony morphology, relevant bio-chemical reactions and serotype.

- The new Colorimetric assay was done for all stool samples to detect the specific enzyme Apyrase. The colorimetric assay was repeated for 141 isolates, which include 138 *E.coli* strains and 3 other organisms that showed positivity in colorimetric assay.

- PCR was carried out for 141 isolates to detect *apy* gene.

- The PCR positive Enteroinvasive *Escherichia Coli* strains were confirmed by sereny test.

- The results of these tests are as follows:

Table-1

AGE AND GENDER DISTRIBUTION OF STUDY AND CONTROL GROUP

In the study group, the male to female ratio was 1:0.9 and among control children the ratio was 1:1.

Table-2

ORGANISMS IDENTIFIED IN STOOL SAMPLES (n=195)

Study group (n=145)			**Control group (n=50)**			**Two sample Binomial proportion test**
No. of samples	Bacterial isolates	Protozoa observed under microscope	No. of samples	Bacterial isolates	Protozoa observed under microscope	z=3.06 p=0.002 Significant
145	166	16	50	74	7	
Total	**182**		**Total**	**81**		

Table-3

TYPE OF ORGANISMS ISOLATED/ VIEWED BY MICROSCOPY FROM DIARRHOEAL STOOL SAMPLE (n=263)

Nature of the isolate	Organism	Study group		Control group	
		No. of the isolates	%	No. of the isolates	%
Bacteria (by culture)	*Escherichia coli*	138	75.82	45	55.55
	Klebsiella species	22	12.08	21	25.92
	Salmonella species	2	1.09	0	-
	Providencia species	2	1.09	1	1.23
	Proteus species	1	0.55	2	2.46
	Citrobacter species	1	0.55	2	2.46
	Pseudomonas species	0	-	3	3.70
Total		**166**	**91.20**	**74**	**91.35**
Protozoa (observed by microscopy)	*Giardia lamblia*	11	6.04	4	4.93
	Entamoeba histolytica	5	2.74	3	3.70
Total		**16**	**8.88**	**7**	**8.64**
Grand total		**182**	**100**	**81**	**100**

Table-4

INCIDENCE OF EIEC IN DIARRHOEAL STOOL SAMPLES BY PCR n= 145

The incidence of EIEC in diarrhoeal stool samples was 5.5%.

Table-5

INCIDENCE OF EIEC AMONG E.COLI ISOLATES BY PCR n= 138

The incidence of EIEC among *E.coli* was 5.79%.

Table-6

COLORIMETRIC ASSAY - APYRASE DETECTION n=145

The enzyme Apyrase detected in 7.58% of study group and none were isolated from the control group.

Table-7

AGE AND GENDER DISTRIBUTION OF EIEC POSITIVE CASES n=8

Age(years)	No. of positive cases	Male		Female	
		No. of cases	percentage	No. of cases	percentage
0-1yr	1	1	12.5	-	-

1-2yrs	4	2	25	2	25
2-3yrs	2	1	12.5	1	12.5
3-4yrs	0	0	-	-	-
4-5yrs	1	1	12.5	-	-
Total	**8**	**5**	**62.5**	**3**	**37.5**

50% of the cases were between 1-2 years of age. The incidence of EIEC was 62.5% in males and 37.5% in females.

Table-8

EIEC ISOLATED FROM CHILDREN WITH DIARRHOEA AND DYSENTERY

n=145

Diarrhoea			**Dysentery**			**Two sample binomial proportion test**
No. of cases	No. of EIEC positive by PCR	%	No. of cases	No. of EIEC positive by PCR	%	Z=9.53 p=0.002 Significant
133	5	3.75	12	3	25	

EIEC was isolated from 3.75% of children with diarrhoea and 25% of children with dysentery.

Table-9

CLINICAL FEATURES OF EIEC POSITIVE CASES n=8

Sl.No	**Clinical features**	**No. of Cases**	**Percentage**
1	Fever	7	87.5

2	Abdominal cramp	6	75
3	Diarrhoea	5	62.5
4	Vomiting	3	37.5
5	Dysentery	3	37.5

EIEC infection presented as diarrhoea in 62.5% of the cases and as dysentery in 37.5% of cases. Fever was the commonest associated symptom.

Table-10

PRESENCE OF LEUKOCYTES AND ERYTHROCYTES IN EIEC POSITIVE STOOL SAMPLE n=8

Symptoms	No. of cases	Leukocytes		Erythrocytes	
		positives	%	positives	%
Diarrhoea	5	3	37.5	2	25
Dysentery	3	3	37.5	3	37.5
Total	8	6	75	5	62.5

75% of cases had leukocytes, 62.5% had erythrocytes in their stool.

Table -11

BIO-CHEMICAL FEATURES OF EIEC n=8

37.5% of the EIEC isolates were non-motile and non-lactose fermenters.

Table –12

OD VALUES AND PCR POSITIVITY IN APYRASE POSITIVE CASES

An OD value of 0.3 was taken as cut-off point.

Table-13

POLYMERASE CHAIN REACTION TARGETING APY GENE (n=141)

5.67 % of the isolates had *apy* gene. The other isolates showed positive Colorimetric assay were negative for PCR.

Table-14

EVALUATION OF COLORIMETRIC ASSAY (n=141)

Colorimetric assay	Polymerase chain reaction		Total
	Positive	Negative	
Positive	8	3	11
Negative	0	130	130
Total	8	133	141

- Sensitivity - 100 %
- Specificity - 98 %
- Positive predictive value - 72 %
- Negative predictive value - 100 %
- Percentage of false positive - 2 %
- Percentage of false negative - 0 %
- Accuracy - 98%
- Kappa - 0.83

Table-15

SERENY TEST PERFORMED ON PCR POSITIVE ISOLATES (n=8)

All the isolates were positive in sereny's test.

Table -16

ANTIMICROBIAL SUSCEPTIBILITY PATTERN (n=8)

All the isolates were sensitive to Amikacin, Cefoperazone-sulbactum and all were resistant to Ampicillin

S : Sensitive

I : Intermediate

R : Resistant

Table -17

ESBL AND MDR PRODUCERS/ MDR STRAINS AMONG EIEC ISOLATES (n=8)

Total No. of isolates	ESBL Production		Multi drug resistance	
	No. of cases	%	No. of cases	%
8	4	50	6	75

50% of the isolates were ESBL producers and 75% were MDR strains

Table-18

SEROTYPING OF EIEC ISOLATES (n=8)

O Serotype	No. of isolates	Percentage
O 28	3	37.5
O 124	1	12.5
O 152	1	12.5
O untypable	3	37.5

The frequently isolated serotype was O28.

DISCUSSION

Acute infectious diarrhoea is one of the major causes of mortality in children, particularly in developing countries like India. EIEC is one of the significant causes of diarrhoea in developing countries. Despite its acknowledged status as a human pathogen, very little research has been conducted.

The culture methods used for the identification of EIEC from stool samples are relatively inefficient. Though molecular methods are rapid and sensitive, they are expensive. The other techniques like animal study and tissue culture are also expensive and time consuming. Hence to overcome the constraints of existing tests, a simple, sensitive and reliable diagnostic test, the “Colorimetric assay” was developed. This study was conducted to evaluate colorimetric assay as a diagnostic test to detect EIEC from stool samples. The results of the study are herewith discussed.

In the present study diarrhoea was common in the age group of 1-2 years of age followed by 2-3 years, 0-1 year, 3-4 years and 4-5 years. In the current study, there was a slightly high proportion of diarrhoea in male children than female children, with the ratio of 1:0.9 and among control children the ratio was 1:1, but statistical analysis by chi-square test showed that the difference is not significant (Table 1). The present study is in

accordance with the study conducted by John Albert, M. et al.,[49].

In this study, 240 bacterial isolates were recovered from 195 stool samples which include 166 strains from study group and 74 strains from control group. Out of 195 samples, protozoans were observed in 23 samples by microscopic examination. Based on statistical analysis of results, significantly higher proportions of organisms were identified in study group (Table 2).

In the present study, a total of 166 strains were isolated from diarrhoeal stool samples, which include *Escherichia coli* (75.82%), *Klebsiella* species (12.08%), *Salmonella* species (1.09%), *Providencia* species (1.09%), *Citrobacter* species (0.55%) and *Proteus* species (0.55%). Cysts of Protozoa were identified in microscopic examination of 16 (8.83%) cases. *Giardia lamblia* was observed in 6.04% of cases and *Entamoeba histolytica* in 2.74% of children with diarrhoea. From the control group, 74 isolates were recovered, of which *E.coli* was isolated in 55.55% of cases. The other bacterial isolates recovered from control group were *Klebsiella* species (25.92%), *Providencia* species (1.23%), *Proteus* species (2.46%), *Citrobacter* species (2.46%), and *Pseudomonas* species (3.70%). The *Giardia lamblia* was observed in 4.93% and *Entamoeba histolytica* in 3.70% of cases (Table-3). A study by Iruka N et al., 2000, has reported *E.coli* in 60.3%, *Klebsiella* species in 30.5%, *Citrobacter* species in 1.2%, and *Salmonella* in

0.9% from stool samples[45]. *Giardia lamblia* was observed in 9.9% of diarrhoeal stool samples by Eneas De Carvalto Aguiar et al., 2006[27]. *Entamoeba histolytica* was observed in 0.6% of 145 children with diarrhoea by John Albert M et al., 1999[49].

In this study, the incidence of EIEC from children with diarrhoea was 5.5%. No EIEC was isolated from control group by colorimetric test (Table 4). Nadia Vieira et al., 2007 reported EIEC from 4.8 % of diarrhoeal children and EIEC in 1.3 % of control children[67]. David N Taylor et al., 1988, reported EIEC from 4% of children with diarrhoea, EIEC from 0.9 % of children without diarrhoea[103]. This is in contrary to the study by Nazek Al-Gallas et al., 2001, which has isolated 11.3% of EIEC from diarrhoeal samples[70]. These findings suggest that the incidence of EIEC might vary in different parts of the world. The statistical analysis of this study shows that with 95% confidence interval the incidence might fluctuate between 2.6-10.3%. In contrast to the present study, Nadia Vieira et al and David N Taylor et al have reported EIEC in the control group, indicating that there is an existence of carrier state in healthy individuals.

The incidence of EIEC among *E.coli* isolates was 5.79%. According to the statistical analysis the incidence might vary from 2 to 11 % (Table 5).

There was a higher incidence of EIEC in the age group of 1-2 years

(50%) followed by 3-4 years of age (25%). The EIEC infection was common among male children (62.5%) than in female children (32.5%) (Table7). This was similar to the study by Elisabeth prestrel et al., 2003, where the incidence of EIEC was higher in children in the age group of 6 months to 2 years[26]. A study by David N. Taylor et al., 1988 disagreed with the present study and has reported increased rate of isolation of EIEC with age and peaked in children of 3-5 years of age[103].

The present study revealed that EIEC was isolated from 3.75% of all cases of diarrhoea and 25% of all cases of dysentery. P value of 0.002 indicates that a significantly higher percentage of dysentery is associated with EIEC infection than diarrhoea (Table 8). A study by David N. Taylor et al., 1988 showed that EIEC was isolated from 4% of all diarrhoeal stool samples and 7% of all dysentery samples[103].

In the present study, 62.5% of patients with EIEC infection had diarrhoea as the presenting symptom, 37.5% of patients presented with dysentery, 87.5 % of patients had fever, 75 % had abdominal cramps and 37 % had vomiting (Table 9). David N Taylor et al., 1988 observed diarrhoea as a presenting symptom in 58.82 %, 35.3 % of patients with EIEC infection had dysentery and the commonest associated symptom with EIEC diarrhoea was fever (90%) followed by vomiting in 47%[103]. The present study correlated well with the study of Nataro et al., 1981, which revealed that EIEC infection

presented most commonly as watery diarrhea and rarely as dysentery[68]. Bui Thi Thu Hien et al reported watery diarrhoea in 100%, fever in 100%, vomiting in 100% and abdominal pain in 50% cases of EIEC diarrhoea[13].

In this study, 75% of patients with EIEC infection had leukocytes in their stools and 62.5% of patients with EIEC infection had erythrocytes in their stools. Only 3 children (37.5%) had dysentery, whereas erythrocytes were observed in 5 (62.5%) of children with EIEC infection, which implies that the remaining 2 (25%) of them might have had occult blood in their stools (Table 10). David N Taylor et al., 1988 reported erythrocytes in 89% and leukocytes in 44% of children with EIEC infection[103]. A study by Echeverria et al 1991 showed erythrocytes and leukocytes in 36% of cases with EIEC infection[25].

In the current study, 37.5% of EIEC were lactose negative and non-motile (Table 11). David et al., 1985, found that 35% of EIEC isolates were lactose negative, 91% were non-motile[103]. Flores Abuxapqui JJ et al reported that 8.33% of EIEC recovered from stool sample were lactose negative[32]. A study by Gustavo Faundez et al., 1988, stated that 85% of EIEC were non-motile and 35% were non-lactose fermenters[39].

Table 12 shows the optical density values and their PCR result in positive cases.

In the study group, of the total stool samples tested, the Colorimetric assay for apyrase was positive in 7.58% (11) cases. None of the stool samples from control group showed positive test in colorimetric assay. Binomial proportion test shows that Apyrase detection was higher in study group than in the control group. (Table 6). At a cut-off value of 0.3, the test showed 97.8% specificity (Table 14), with 3 false positives that may be due to *Providencia* species (2) and *Proteus* species (1). The reasons for false positives are unknown, but PCR analysis indicated that none carried the *apy* gene (Table13). Furthermore, not all isolates of these species were positive.

A total of 141 isolates were tested for apy gene by PCR, 8 of them exhibited positive band and there was no false positive by PCR. With 95% confidence interval detection of *apy* gene by PCR possibly will vary from 2.6-10.5% (Table 13). Krishnan Sankaran et al., 2008 stated that PCR targeting *apy* gene was 100% sensitive in detecting EIEC infection[55].

The sereny test was done on 8 PCR positive isolates and all were positive (Table 15). This test proves the invasive nature of isolates and confirms EIEC. Thus among *E.coli* only Enteroinvasive *E.coli* will express apyrase enzyme. Sensitivity of the test was analogous with the studies by Flores Abuxapqui JJ et al., 1999[32], and Nazek Al-Gallas et al., 2007[70].

Considering PCR as a gold standard test for EIEC, the sensitivity and specificity of colorimetric assay in our study was 100% and 97.8% respectively. The percentage of false positive was 2.1% and there was no false negative. The accuracy of the test was 98%; this may perhaps vary from 94-100% (Table 14).

All the EIEC isolates were sensitive to Amikacin and Cefoperazone-Sulbactum. 62.5% of the isolates were sensitive to Ciprofloxacin, 37.5% of the isolates to Cefotaxime, 12.5% isolates to Co-trimoxazole, 50% isolates to Gentamicin and none were sensitive to the most commonly used antibiotic Ampicillin (Table 16). Resistance to ≥ 3 drugs was observed in 75% of EIEC isolates and ESBL production was seen in 50 % of EIEC isolates (Table 17).

The antibiotics Amikacin and Cefoperazone-Sulbactum were active against all isolates, but these can only be administered in hospital settings. The strains showed 75% sensitivity to Ciprofloxacin; however Ciprofloxacin and other quinolones are not approved for children. All the isolates showed 100% resistance to Ampicillin and 87.5% to Co-trimoxazole. This could be due to the reality that, most of the cases admitted in the wards could be refractory to treatment, since this is a tertiary care hospital. However results of the current study might not be statistically significant, because, lesser number of EIEC isolates were analyzed.

The study by Teresa Estrada et al., 2005 on antimicrobial susceptibility pattern of EIEC revealed resistance of 50% to ampicillin and all were sensitive to co-trimoxazole. 50% of the isolates showed multi drug resistance i.e. resistance to Ampicillin, co-trimoxazole and chloramphenicol[104].

In the present study, frequently isolated serotype was O28 (37.5 %). 12.5 % of the isolates belonged to O124, 12.5 % to O152 and 37.5 % were untypable (Table 18). O28ac (25 %), O124 (25 %) were the frequently isolated serotypes in the study conducted by Gustavo Faundez et al., 1988[39]. Prats G et al., quoted that O124 was the most frequently isolated serotype.[80]

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SUMMARY

- Totally 195 children 0-5 years of age with diarrhoea and without diarrhoea were included in the study.
- This study was done to evaluate colorimetric assay as a diagnostic test to detect EIEC.
- Colorimetric assay and PCR were done for all the samples to detect Apyrase enzyme and *apy* gene respectively, which are specific for EIEC.
- Incidence of EIEC in children with diarrhoea was 5.5 %.
- EIEC infection presented most commonly as diarrhoea (75%) and rarely as dysentery (25%). The commonest associated symptom was fever followed by abdominal pain and vomiting.
- Incidence of EIEC infection was higher in the 1-2 years of age (50%) followed by in 2-3 years of age (25%).
- There was preponderance of EIEC infection in males (62.5%).
- Majority of the EIEC isolates were lactose positive (75%).
- Out of 145 samples tested, colorimetric assay for the enzyme Apyrase was positive in 7.58 % cases of diarrhoea. Colorimetric assay had 100 % sensitivity and 97.8% specificity. This test overcomes many of the constraints of standard available methods like expensive equipment, need for well trained hands, cost of maintenance of the animals and limited availability in developing countries. Hence, the excellent sensitivity of this test can be made use of in diagnosing pathogenic strains of EIEC from diarrhoeal stool samples.

- PCR for *apy* gene was positive in 5.5% of cases.
- Colorimetric assay correlated well with the Sereny's test.
- All the EIEC isolates were sensitive to Amikacin and Cefoperazone-Sulbactum and all were resistant to Ampicillin. The isolates showed variable resistance to Ciprofloxacin (12.5%), Co-trimoxazole (75%), Gentamicin (50%). 75% of the isolates showed multidrug resistance.
- 50 % of the EIEC isolates were ESBL producer.
- O28 was the frequently isolated serotype.

CONCLUSION

Incidence of EIEC in children with diarrhoea was 5.5%. Evaluation of colorimetric assay shows that this can be adopted as a diagnostic test to detect Apyrase enzyme of EIEC from stool samples. Besides this test is simple, less expensive and results can be obtained earlier, to start antibiotics promptly. Therefore further complications can be prevented. PCR targeting *apy* gene is more specific for diagnosing EIEC infection. Antibiogram should be done for all dysentery cases, since most of the strains showed multidrug resistance (75%) and ESBL production (50%). Amikacin and Cefoperazone-Sulbactum are recommended for severe cases of diarrhoea. O28 was frequently isolated serotype.

Any disease for that matter *"Prevention is always better than Cure"*. This can be achieved by promotion of exclusive breast feeding, improved water sanitation and promotion of personal and environmental hygiene.

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APPENDIX I

Mac Conkey agar

Peptone	20g
Sodium taurocholate	5g
Agar	20g
Neutral red solution, 2% in 50% ethanol	3.5g
Lactose, 10 % aqueous solution	100ml

Dissolve the peptone and taurocholate in the water by heating. Add the agar and dissolve it in the steamer or autoclave. If necessary, clear by filtration. Adjust the pH 7.5.

Xylose lysine deoxycholate agar

Yeast extract	3g
Xylose	3.75g
Lactose	7.5g
Sucrose	7.5g
L-Lysine	5.0g
Sodium Chloride	5.0g
Sodium deoxycholate	2.5g
Sodium thiosulphate	6.8g
Ferric ammonium citrate	0.8g
Phenol red	0.08g
Agar	15 g
Water	1 Litre

Except for the deoxycholate, thiosulphate and ferric ammonium citrate, dissolve the ingredients in the water by autoclaving. Cool to 50˚c and add 20ml of a solution of sodium thiosulphate 34% and ferric ammonium citrate 4%, and 25 ml of a solution of sodium deoxycholate 10 %. Adjust the pH to 7.4.

Selenite F broth

Sodium hydrogen selenite	4 g
Peptone	5 g

Lactose	4 g
Disodium hydrogen phosphate	9.5 g
Sodium dihydrogen phosphate	0.5 g
Sterile water	1 litre

All the ingredients were dissolved in sterile water with sterile precautions. The yellow solution in about 10ml amounts distributed into screw capped universal bottles. Steamed for 20 minutes at 100˚c. Adjust pH to 7.1.

Mueller- Hinton Agar:

Beef extract	20 gm
Acidicase peptone	7.5 gm
Starch	1.5 gm
Agar	17 gm
Distilled water	1000 ml

The ingredients were dissolved in one liter of distilled water, mixed thoroughly. Heated with frequent agitation and boiled for 1 minute. pH adjusted to 7.4. Sterilized by autoclaving and poured in plates.

APPENDIX II

Catalase

3 % hydrogen peroxide

Oxidase reagent

1% Tetra methyl para- phanelyne diamino dihydrochloride

Kovac's reagent

Para-dimethyl amino benzaldehyde	10gm
Iso-amyl alcohol	150 ml
Concentrated – Hydrochloric Acid	80 ml

Triple Sugar Iron

Peptone	2 gm
Sodium Chloride	0.5gm
Yeast Extract	0.3gm
Beef Extract	0.3gm
Glucose	0.1gm
Lactose	1.0gm
Sucrose	0.2gm
Ferric Citrate	0.03gm
Sodium thiosulphate	0.03gm
Sodium Chloride	0.5 gm
Phenol Red 0.2% Solution	1.2ml
Distilled Water	100 ml
pH	7.4

Heat to dissolve the solids. Add the indicator solution. Mix and sterilize at 121˚C for 15 minutes and cool to form slopes with deep (3cm)butts.

Simmons citrate agar

Ammonium dihydrogen phosphate	0.1gm
Potassium dihydrogen phosphate	0.1gm

Sodium chloride		0.5gm
Sodium citrate		0.5gm
Magnesium sulphate	-	0.02gm
Bromothymol blue 0.2%		4ml
Agar		2gm
Distilled water		100 ml
pH		6.8

Dispense and autoclave at 121˚c for 15 minutes and allow cooling to form slopes.

Christensen's urea agar

Peptone	0.1gm
Glucose 10% solution	1ml
Sodium chloride	0.5gm
Dipotassium hydrogen phosphate	0.2gm
Agar	2gm
Distilled water	100ml

Sterilize the glucose and urea solution by filtration.Prepare the basal medium without glucose or urea, adjust to pH 6.8-6.9 and sterilize by autoclaving in a flask at 121˚c for 30 minutes. Cool to about 50˚c, add glucose, urea and form slopes with deep

APPENDIX III

REAGENTS REQUIRED FOR THE COLORIMETRIC ASSAY

WASH REAGENT

Saline-$CaCl_2$: 0.9% NaCl containing 1mM $CaCl_2$

ASSAY REAGENT

Assay Buffer: 40mM EDTA, pH 7.5

1.5g of Di sodium EDTA is dissolved in 80 ml of phosphate-free distilled water. This is a stable reagent.

Substrate Solution: 6mM Sodium Pyrophosphate

1.6 mg of Sodium Pyrophosphate decahydrate is dissolved in 1ml of phosphate-free distilled water.

Just before assay, mix equal volumes of Assay Buffer and substrate solution to give the final reagent, which is 3mM Sodium Pyrophosphate in 20mM EDTA, pH 7.5.

COLOR REAGENT

Reagent A: 5% Acidic Ammonium Molybdate

5 g of Ammonium Molybdate is dissolved gradually in 10N H_2SO_4.

Reagent B: 1% Ferrous Ammonium sulphate (FAS)

1 gram of FAS is weighed and dissolved in 10 ml of phosphate free distilled water.

LURIA BERTANI BROTH

Sodium Chloride	:	10g
Tryptone	:	10g
Yeast Extract	:	5gm
Distilled Water	:	1 Litre
pH	:	7.2

The ingredients were dissolved in one liter of distilled water, mixed thoroughly. Heated with frequent agitation and boiled for 1 minute. pH adjusted to 7.4. Sterilized by autoclaving and poured in plates.